High-Level Expression, Purification, and Characterization of the Recombinant Grass Carp Pituitary Adenylate Cyclase-Activating Polypeptide

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Pituitary adenylate cyclase-activating polypeptide-38 (PACAP38) is a potent secretagog for growth hormone and gonadotropin in fish species. To obtain recombinant grass carp PACAP38, its open reading frame was subcloned in pET32a(+) vector to express thioredoxin (Trx)-PACAP fusion protein in *Escherichia coli* BL21 (DE3). The resulting expression level of the thioredoxin-PACAP reached 36% of the total proteins, and more than 85% of fusion protein existed as soluble form. Using Ni²⁺-chelating affinity chromatography, 102 mg of Trx-PACAP38 with a purity of 97% was obtained from 342 mg of crude proteins from a 1-liter culture of *Escherichia coli*. The purified Trx-PACAP specifically inhibited T98G human glioblastoma cell proliferation, but the fusion partner had no effect in this regard. Moreover, this inhibition was totally abolished by PACAP-specific antibody.

Key words: recombinant pituitary adenylate cyclase-activating polypeptide (PACAP); fusion expression; purification; biological activity

Pituitary adenylate cyclase-activating polypeptide (PACAP), a member of the secretin/glucagon/vasoactive intestinal peptide (VIP) superfamily, was first identified in ovine hypothalamus by its stimulatory effect on cAMP production in rat pituitary cells. This peptide exists in two forms, PACAP38 and PACAP27, the latter being the N-terminal portion of PACAP38. Both forms of PACAP act by binding to their G protein-coupled receptors: PAC1, VPAC1, and VPAC2. Activation of multiple receptors by PACAP has broad physiological effects on the nervous, endocrine, reproductive, cardiovascular, muscular, and immune systems.

In teleost fish, PACAP immunoreactivity can be detected in different parts of the pituitary. In goldfish, PACAP fibers are present in the pars distalis and neurointermediate lobe of the pituitary, and more recently, nerve fibers with PACAP immunoreactivity were found to overlap with the distribution of somatotrophs in the grass carp pituitary. The presence of a PACAP neuronal system within the pituitary is consistent with findings that PACAP can act as a potent secretagog for GH secretion in salmon, goldfish, European eel, common carp, and grass carp. Notably in goldfish, both PACAP27 and PACAP38 were effective in stimulating GH secretion through activation of the adenylate cyclase-cAMP-protein kinase A and phospholipase C-IP3-protein kinase C pathways in cultured pituitary cells. In another teleost grass carp, two forms of ovine PACAP not only trigger GH release but also enhance GH synthesis at the pituitary cell level. In the same animal model, a full-length cDNA of PACAP has been cloned and confirmed to be a single-copy gene in the genome. Similarly to ovine PACAP, two synthetic forms of grass carp PACAP were effective in elevating GH release and GH transcripts in pituitary cells, and PACAP38 was consistently found to be more potent in GH secretion than PACAP27. In mammals, GH secretion is primarily under the stimulatory control of GHRH, while studies aimed at the effect of PACAP on GH secretion have indicated that PACAP probably plays a minor role in the control of GH secretion. Recently, non-mammal GHRH peptides encoded in cDNAs isolated from goldfish, zebrafish, and frog were identified. In the goldfish, a 4-h incubation of pituitary cells with increasing doses (0.1 nM–1 μM) of goldfish GHRH resulted in a dose-dependent increase in GH release, the minimum effective dose being 10 nM. Like GHRH, PACAP38 (0.1 nM–1 μM) was also effective in inducing GH release from both goldfish and grass carp perifused pituitary cells in a dose-dependent manner. These findings indicate that both PACAP and GHRH are...
potent GH-releasing factors in teleost fish, and suggest that PACAP can be applied to increase yields in commercial fishery.

The relationship between PACAP and food intake is still a matter of heated debate. PACAP has been reported to inhibit food intake during 60 min of observation in goldfish, as in mice and chickens, which might compromise the growth-promoting action resulting from PACAP-induced GH secretion. However, a more recent study indicated that PACAP is not required for the regulation of food intake in PACAP-null mice. The reason for this discrepancy is still unknown, but might be species-specific variability and/or differences in research methodology. Moreover, it is still unclear how PACAP regulates appetite in the long term. Since the results of GH administration and GH transgenesis stimulating body growth might be attributable to increased feeding and improved food assimilation in fish, we do not exclude the possibility that PACAP administration optimized to stimulate GH release can lead to increased body growth. Possible additional roles of PACAP have been confirmed in fish models, lead to increased body growth. Possible additional roles in fish life. Consequently, mass preparation of recombinant grass carp PACAP has been confirmed in fish models, especially in inducing gonadotropin release in goldfish, and a developmental role in zebrafish, indicating that PACAP, alone or in concert with other regulators, plays different roles in fish life. Consequently, mass preparation of recombinant grass carp PACAP can be used in the improvement of fish culture, and allows further in vitro and possibly in vivo studies to determine the physiological roles of PACAP in commercial fish.

Chemical synthesis is an option for producing PACAP, but the technique encounters potential problems. First, peptide synthesis of over 35 amino acids is generally not economically feasible, since the chemical synthesis of longer peptides (more than 50 amino acids) is technically challenging. In addition, the process of chemical synthesis is step-intensive, and does not serve well for large-scale production. Alternatively, recombinant DNA technology provides a ready means of greater production yields at decreased cost, and reduces the use of hazardous materials. In the present study, an efficient method of producing recombinant PACAP38 of grass carp was developed. Fusion of grass carp PACAP38 to thioredoxin (Trx) not only facilitated the high expression and accumulation of fusion proteins in soluble form, but also simplified the purification procedures. Notably, small peptides (<5 kDa) are rapidly degraded and thus can have extremely short half-lives. In this regard, fusion peptide can enhance its stability. In this study, deoxyoligonucleotide encoding grass carp PACAP38 was designed according to the codon preference of E. coli, which improved the translation efficiency of grass carp PACAP in the E. coli expression system.

Materials and Methods

Strains and plasmids. Escherichia coli (E. coli) strains JM109 and BL21 (DE3) were used as host cells in subcloning and PACAP fusion protein expression respectively. pUCm-T vector was purchased from Shenergy Biocolor (Shanghai, China). pET32a(+) vector was obtained from Novagen (Madison, WI). Other reagents were from commercial resources, and were of high quality.

Construction of pET32a(+)/PACAP expression plasmid. The amino acid sequence of grass carp PACAP38 was obtained from GenBank (accession no. ABQ81649), and was back-translated to the DNA sequence using E. coli preferential codons. To effectively terminate the translation, two continuous termination codons were supplemented to the 3′ end of this DNA fragment. The PACAP open reading frame (ORF) was synthesized and amplified by a two-step strategy with three oligonucleotides (Fig. 1). A pair of restriction endonuclease (BamHI and HindIII) sites flanked the 5′ upstream and 3′ downstream of PACAP ORF respectively. First, a chain extension reaction was completed with oligonucleotide F1 (5′-GCAGATCCATTTACAGCCGCTACCGTAAACAGATG-3′) and oligonucleotide R1 (5′-ACGACGACCCGAGCTGCTGAATATCTTCTTGACGGCCATCTGTTTACGGTAGCGGCT-3′), which were denatured at 94°C for 10 min following 10 min of annealing at 55°C. Then Taq DNA polymerase was added, and extension was carried out at 72°C for 5 min. Secondly, the PACAP fragment was amplified using chain extension reaction product as template, and oligonucleotide F1 and oligonucleotide R2 (GCAAAGCTTCTCATA-TTTGTTTTTTATGCGCTGGGCGGTACACGGACCCGAC-CCAGGGGCTG) as primers. The conditions for PCR amplification were as follows: 94°C for 3 min; 30 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s, and a final extension for an extra 10 min at 72°C. After PCR, the product was digested with BamHI and HindIII, and the digested DNA fragment was inserted into BamHI-I-HindIII digested pET32a(+) vector to form a PACAP expression plasmid named pET32a(+)/PACAP. The sequence of PACAP ORF in this plasmid was confirmed by DNA sequencing (ABI PRISM 3730 sequencer, Applied Biosystems, Foster City, CA).

Fusion protein expression. After pET32a(+)/PACAP was transformed into E. coli BL21 (DE3) cells, a pilot experiment was done to optimize the time and temperature for the expression of thioredoxin-PACAP (Trx-PACAP) fusion protein. The clone of interest was streaked onto a Luria-Bertani (LB) plate containing 100 μg/ml of ampicillin, and incubated at 37°C overnight. The single colony selected harboring the plasmid was cultured in 5 ml of LB medium containing 100 μg/ml of ampicillin, and incubated at 37°C with shaking at 210 rpm (Thermo 481, Boston, MA). Overnight culture (2.5 ml) was used to inoculate 50 ml of fresh LB medium in a 250-ml culture flask, and we grew this culture at 30°C until the OD600 was between 0.6 and 0.8.
Expression of the fusion protein was induced with isopropyl-β-D-thiogalactoside (IPTG, Merck, Darmstadt, Germany) at a final concentration of 1 mM. To perform time-course expression, 1-ml cultures were sampled at 2-h intervals after induction for up to 8 h. Cells were collected by centrifugation for 3 min at 13,200 rpm (Eppendorf 5415, Hamberg, Germany) and stored at -20°C. After the supernatant was decanted, cell pellets were resuspended in 500 ml of ice-cold imidazole buffer (20 mM PB, pH 6.6, 2.5 mM EDTA, and 5 mM imidazole), and kept on ice. Then each sample was sonicated in 10-s bursts 3 times using a hand-held sonicator (Cole-Parmer, Vernon Hills, IL) with a microtip. These samples were snap-frozen at -80°C freezer and quickly thawed at 37°C. The sonication-freeze-thaw cycle was repeated 3 times. Cell lysate was centrifuged at 13,200 rpm at 4°C for 30 min to pellet cell debris and insoluble matter. The supernatant was transferred to new tubes, and the pellet was resuspended in 500 μl of ice-cold imidazole buffer. Finally, each sample was analyzed with SDS–PAGE gel (15% separating gel and 4% stacking gel).

**Purification of fusion protein.** For PACAP fusion protein purification, E. coli BL21(DE3) cells transformed with pET32a(+) PACAP plasmid were cultured in 50 ml of LB medium containing 100 μg/ml of ampicillin overnight at 30°C at 210 rpm. In a parallel experiment, pET32a(+) vector was transformed into BL21(DE3) cells, and this served as a negative control. Then the culture was transferred to 1 liter of fresh LB medium with 100 μg/ml of ampicillin, and incubated at

**Fig. 1.** Construction of Trx-PACAP Fusion Protein Expression Plasmid.

A, Modified grass carp PACAP38 open reading frame and corresponding amino acids. The nucleotides, modified according to the codon preference of E. coli, are shadowed with dark gray. The oligonucleotides used in PCR are indicated by an arrowhead line. The restriction enzyme cut sites are noted as black triangles. B, Strategy for Trx-PACAP expression vector construction. Oligonucleotide encoding grass carp PACAP was prepared by two-step PCR, and the PCR product was subcloned into bacteria expression vector pET32a (+) by BamH I and Hind III digestion.
30°C until the OD_{600} was between 0.6 and 0.8. Then 1 mM IPTG was added for an additional 4 h. Cells were harvested by centrifugation at 3,000 g at 4°C for 10 min. The harvested cells were resuspended in 40 ml of buffer A (20 mM imidazole, 500 mM NaCl, and 20 mM phosphate buffer, PB, pH 6.6) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF, Amresco, Solon, OH), 1 mM MgCl₂, and 0.2 mg/ml lysozyme. The suspension was incubated for 30 min at room temperature with a gentle vortex and sonicated at 500 watts (25%) for 50 cycles (5 s working, 10 s free). The cell lysate was clarified by centrifugation at 13,200 rpm for 30 min at 4°C.

The clarified supernatant was loaded in an IMAC (Imobilized Metal Affinity Chromatography) column HiTrap FF (Amersham Pharmacia Bio-Science, Uppsala, Sweden) packed with 1.0 ml of nickel-iminodiacetic acid resin equilibrated with 5 column volumes of buffer A. Buffer A and buffer B (50 mM imidazole, 500 mM NaCl, and 20 mM PB, pH 6.6) were applied at a flow rate of 1 ml/min to remove unbound proteins in turn. Bound proteins were eluted with buffer C (500 mM imidazole, 500 mM NaCl, and 20 mM PB, pH 6.6), and the eluted protein was desalted with a desalting column (Sephadex G-25) in buffer D (20 mM PB, pH 6.6) at 4 ml/min. Affinity chromatography and desalting proceedings were run on A¨KTA Explorer100 FPLC (Amersham Biosciences, Uppsala, Sweden). Eluted protein was monitored by OD at 280 nm, and the protein concentration was determined by Bradford assay with bovine serum albumin (Sigma, St. Louis, MO) as the standard. Finally, the fractions were collected and applied to 15% SDS–PAGE. After Coomassie Brilliant Blue staining, protein bands were analyzed by optic densitometry using Quantity One software (Bio-Rad, Hercules, CA).

In parallel experiments, the fusion partner (including Trx, the enterokinase site, and the multiple cloning site) was expressed and purified by methods similar to those described above. In this case, incubation of E. coli BL21(DE3) cells containing blank vector was noted at 37°C but not at 30°C.

**Western blot assay.** Protein samples were separated by 15% (w/v) SDS–PAGE and transferred to PVDF membrane (Millipore, Billerica, MA) using a semi-dry transfer apparatus (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. After incubation with 5% (w/v) non-fat milk in TBS (10 mM Tris–HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.4) for 4 h at room temperature, the membrane was incubated with rabbit anti-PCAP-27/38 polyclonal antibody (Bios, Beijing, China) at 1:300 dilution with gentle agitation overnight at 4°C. After a brief washing step, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (ZSGB-BIO, Beijing, China) at 1:10,000 dilution with gentle agitation for 1 h at room temperature. Finally, protein was visualized with an enhanced horseradish peroxidase-diaminobenzidine substrate kit (Tiangen Biotech, Beijing, China).

**Bioactivity assay of Trx-PACAP fusion protein.** To test the biological activity of Trx-PACAP, the effect of Trx-PACAP on T98G human glioblastoma cell proliferation was examined. In this case, T98G cells (1 × 10^5 cells/well) were cultured in a 24-well plate (Becton Dickinson, Franklin Lakes, NJ) in 0.5 ml of RPM-1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (PAA, Haidmannweg, Germany). After the cells were recovered at 37°C under 5% CO₂ and saturated humidity for 24 h, the time-course effect of the Trx-PACAP fusion protein was determined by incubation with the cells for 48, 72, and 96 h. For dose-dependent studies, T98G cells were incubated with increasing concentrations of either the Trx fusion partner or the Trx-PACAP fusion protein for 72 h. In this experiment, the effect of the fusion protein on T98G proliferation was determined with a WST-8 4-[3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate sodium salt) assay kit (Beyotime Institute of Biotechnology, Haimen, China). Briefly, 100 µl of Cell Counting Kit solution was added to each well after treatment. The plates were then incubated for additional 3 h, and 100 µl of completely mixed solution of each sample was collected into a 96-well plate. The absorbance was measured with a Model 680 micro-plate reader (Bio-Rad, Hercules, CA) at 450 nm with a reference wavelength of 630 nm. Cell viability was calculated by the following formula:

\[
\text{Cell Viability} = \frac{\text{OD}_{450} (\text{sample}) - \text{OD}_{630} (\text{sample})}{\text{OD}_{450} (\text{control}) - \text{OD}_{630} (\text{control})}.
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**Results and Discussion**

**Construction of the plasmid and expression of the fusion protein.** To improve translation efficiency, oligonucleotides for PACAP₃₈ ORF synthesis and amplification were designed based on the E. coli preferred codons (Fig. 1A). The DNA fragment was subcloned into pET32a(+) vector, resulting in a Trx-PACAP fusion protein expression plasmid, pET32a(+)/PACAP (Fig. 1B). In this study, the BL21(DE3)/pET32a(+) system was selected for the following reasons: both the ompT and Lon proteases are deficient in the BL21(DE3) strain, which are important proteases responsible for the degradation of heterologous proteins; in tDE3 lysogen, the T₇ RNA polymerase gene is under the control of the lacUV5 promoter, which protects the host cells from the toxicity of the Trx-PACAP fusion protein before IPTG induction; the T₇lac promoter can also be induced by IPTG to initiate fusion protein expression. Thus the Trx-PACAP fusion protein could be expressed at a high level in the E. coli expression system.
To test the time-course effect of IPTG induction on Trx-PACAP fusion protein expression, samples from the supernatant or the pellet of cell lysate were analyzed by SDS–PAGE after the addition of 1 mM IPTG for durations as indicated above. Compared with the sample without IPTG induction, an extra 23-kDa band was detected in the total protein sample from the supernatant after 2, 4, 6, and 8 h of induction by IPTG. The size of this new protein was in accordance with the size of the Trx-PACAP fusion protein (22.4 kDa, calculated from the amino acid sequence), and its expression reached a maximum after 4 h of induction (Fig. 2A). In a parallel experiment, the new protein in the pellet was significantly lower in quantity than that in the supernatant (Fig. 2B), indicating that the fusion protein was mainly retained in the supernatant in a soluble form (> 85%). Apparently, fusion of a small peptide to Trx is helpful in improving protein yield and solubility. Notably, the expression level of the 23-kDa soluble protein at 37°C was significantly less than at 30°C, but the fusion partner was highly expressed after induction at 37°C (data not shown).

**Purification of the fusion protein**

Large scale expression of fusion protein was performed in 1 liter of culture following the procedures described above. After centrifugation, a Bradford assay showed that a total of 342 mg of soluble protein was obtained from the cell lysate. The fusion protein in the supernatant was then purified by Ni²⁺-chelating chromatography based on the hexa-histidine tag in the fusion protein. SDS–PAGE analysis revealed the effectiveness of this one-step purification (Fig. 3). Undesirable proteins caused to flow through the column or were washed by 50 mM imidazole, and the majority of fusion protein was eluted by 500 mM imidazole. Densitometry analysis using Quantity One software showed that the fusion protein reached more than 36% of the total protein.
and the purity of the fusion protein reached 97% or more (Fig. 3A, lane 4). The amount of fusion protein was 123 mg in the supernatant (Table 1). Finally, a total of 99 mg of fusion protein was obtained through purification and desaltification, indicating that the yield of fusion protein was 80% (Table 1). To confirm the identity of the expressed fusion protein, Western blot analysis was performed. It showed that the purified protein was specially recognized by anti-PACAP-27/38 antibody (Fig. 3B).

Characterization of Trx-PACAP fusion protein

Given that the amino acid sequence of grass carp PACAP<sub>38</sub> is highly homologous to sequences reported for other species<sup>11)</sup> and that PACAP receptors were present in T98G human glioblastoma cell line,<sup>25)</sup> we used this cell line to test the biological activity of grass carp PACAP fusion protein. Time-course studies showed that 100 nM Trx-PACAP fusion protein markedly inhibited T98G cell proliferation after treatment for 48 h, 72 h, and 96 h as compared with the time-matched controls (fusion partners) (Fig. 4A). The time point corresponding to maximal effect was 72 h (Fig. 4A). This inhibitory action was consistently observed in the same cells exposed to human PACAP. On the other hand, a 72-h incubation of T98G cells with increasing concentrations of Trx-PACAP fusion protein (0.1–1,000 nM) resulted in a dose-dependent decrease in cell proliferation. The minimal dose of fusion protein tested triggering a drop in cell proliferation was noted at 1 nM (Fig. 4B). In parallel experiments, the Trx fusion partner did not alter T98G cell proliferation in this regard (Fig. 4B). To further confirm that the effect of the fusion protein on cell proliferation was specifically caused by PACAP, PACAP immunoneutralization was performed. In this case, the responsiveness of T98G cells to Trx-PACAP was tested in the presence of anti-PACAP-27/38 antibody (Fig. 4C). The results showed that the inhibitory effect of Trx-PACAP was totally abolished by anti-PACAP-27/38 antibody at 1:1,000 and 1:300 dilutions (Fig. 4C). These results indicate that the fusion partner did not affect the function of PACAP. They are in agreement with a report that prion protein fused to blue fluorescent protein retained its biological activity. Another finding also showed that the presence of human serum albumin (fusion part) on the C-terminus of glucagon-like peptide-1 did not affect peptide activity, and that the fusion protein presented a longer action time than the native peptide.<sup>27)</sup>

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**Table 1.** Purification of Trx-PACAP Fusion Protein from 1 Liter E. coli Culture

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total protein&lt;sup&gt;a&lt;/sup&gt; (mg)</th>
<th>Purity&lt;sup&gt;b&lt;/sup&gt; (%)</th>
<th>Object protein&lt;sup&gt;c&lt;/sup&gt; (mg)</th>
<th>Yield&lt;sup&gt;d&lt;/sup&gt; (%)</th>
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<tbody>
<tr>
<td>Cell lysate</td>
<td>342</td>
<td>36</td>
<td>123</td>
<td>100</td>
</tr>
<tr>
<td>IMAC + Desalting</td>
<td>102</td>
<td>97</td>
<td>99</td>
<td>80&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
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<sup>a</sup>Total protein concentration was determined by Bradford protein assay, using bovine serum albumin as a standard.

<sup>b</sup>Purity was determined by densitometry scanning of Coomassie Blue stained SDS–PAGE using Quantity One software (Bio-Rad).

<sup>c</sup>The quantity of object protein was calculated based on the % purity.

<sup>d</sup>The purification yield was calculated based on the amount of object protein.

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![Fig. 4.](image-url) Effects of Trx-PACAP Fusion Protein on T98G Human Glioblastoma Cell Proliferation.

A. Time-course effects of Trx-PACAP on T98G cell proliferation. T98G cells were incubated with 100 nM fusion partner or Trx-PACAP for 48, 72, and 96 h. B. Dose-dependent effects of Trx-PACAP and of the fusion partner on T98G cell proliferation. Cells were incubated with increasing doses of Trx-PACAP and of the fusion partner for 72 h. C. Effects of PACAP antibody on Trx-PACAP inhibited T98G cell proliferation. T98G cells were incubated with 100 nM Trx-PACAP supplemented with 1:300 or 1:1,000 PACAP antibody or not for 72 h. Cell proliferation was measured by WST-8 assay. Data presented (mean ± SEM, N = 5) are pooled results from three separate experiments. Asterisks denote significant difference (P < 0.05).
In conclusion, a grass carp PACAP<sub>38</sub> bacterial expression plasmid was constructed according to the codon preference of E. coli using a two-step PCR strategy. A high yield of soluble Trx-PACAP protein (123 mg/l) was obtained using a BL21 (DE3) E. coli expression system, and a large quantity of recombinant PACAP (99 mg/l) was purified with an IMAC column. Moreover, the biological activity of this peptide was confirmed by inhibiting T98G human glioblastoma cell proliferation. This PACAP preparation method is simpler and more cost-effective for large-scale production than chemical synthesis. Significant quantities of recombinant grass carp PACAP have the potential to facilitate analysis of physiological role of PACAP in fish, as well as to facilitate the development of applications to fish culture in regard to increasing evidence of PACAP-inducing GH release in fish models.

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References


